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EMBRYONIC DEVELOPMENT OF AN ENDOPARASITOID, *MICROPLITIS CROCEIPES* (HYMENOPTERA: BRACONIDAE) IN CELL LINE-CONDITIONED MEDIA

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SUMMARY

Embryos of the parasitoid *Microplitis croceipes* develop from pregerm band stage to first larval instar in cell culture medium conditioned by a cell line (IPLB-LdFB) derived from fat body from an atypical host *Lymantria dispar*. However, the percentage of eggs that develop normally to the first larval instar stage is significantly less than for those maintained in IPL-52B medium conditioned with host fat body tissue. Therefore, we examined the capacity of five insect cell lines to promote growth and development of pregerm band eggs in five media, IPL-52B, TC-199, TC-100, Grace's, and ExCell 400. The developmental response of *M. croceipes* was dependent both on the cell line and the cell culture medium used. TC-100, TC-199, and Grace's media promoted development to the germ band stage without the need for conditioning with host tissue. IPL-52B supported development to the germ band stage when a defined lipid concentrate was added. In IPL-52B medium, the IPLB-LdFB cell line promoted a significantly higher number of eggs developing to germ band relative to the other cell lines; however, none of the cell line-conditioned IPL-52B medium significantly stimulated egg hatch relative to the control medium. None of the cell line-conditioned Grace's media had a significant effect on eggs attaining germ band stage compared with the Grace's control medium. However, Grace's medium conditioned with the IAL-TND1 and IPLB-LdFB cell lines promoted development beyond germ band, resulting in a significantly higher percentage of hatching eggs than the Grace's control medium. Although the BCIRL-HZ-AM1 cell line, which is derived from the parasitoid's typical host, did not induce hatch in either IPL-52B medium or Grace's medium, it promoted hatch in TC-199 and Excell 400 media. Fat body taken from the same species that the cell lines were derived from was a better predictor of a cell line's embryotrophic activity in Grace's medium rather than in IPL-52B medium. Thus, the composition of the medium and the species and tissue type of the cell line source must be evaluated interactively to determine optimal conditions for promoting development of *M. croceipes* in vitro.

Key words: cell line; media; germ band; larval; instar; fat body; hatch; lipid.

INTRODUCTION

The mass-rearing of insect parasitoids in vitro is of interest in agriculture because of the potential use of the technology in biological control of important insect pests. Developing an artificial medium that would allow the parasite to live outside the host would simplify mass-rearing and make production of the parasites economically feasible for release in the field. Although several insect parasites have been successfully cultured on semidefined and defined media, none of the hymenopteran larval endoparasites have been reared from egg to the adult in vitro (7,17,19). Eggs of endoparasitic Hymenoptera differ from those of free-living insects in that they develop in a milieu of hemolymph in the host (4). Eggs of the endoparasitoid *Microplitis croceipes* explanted from the host in the pregerm band stage will not develop in conventional tissue culture media in vitro unless the medium is either supplemented with host hemolymph or conditioned with host fat body tissue (7). In addition, we discovered that conditioned medium from two cell lines, IAL-TND1 and IPLB-LdFB, stimulated development of eggs to the pregerm band stage or to hatching, respectively (2). In the present study, we evaluate several cell lines under a variety of culture conditions for their ability to stimulate development of *M. croceipes*.

MATERIALS AND METHODS

Host and parasite colony maintenance. The host species *H. zea* was mass-reared in our laboratory according to previously described procedures (9). Rearing procedures for *M. croceipes* were as previously reported (3).

Basal medium preparation. Goodwin's IPL-52B medium, a chemically defined cultural medium (6), was prepared as described earlier (2). Excell 400, a serum-free insect cell culture medium was obtained from JHR Biosciences, Woodland, CA. A modified Grace's medium (GIBCO, Grand Island, NY) containing whole egg ultrafiltrate (10%), fetal bovine serum (7%), and bovine serum albumin fraction V (1%) was prepared according to Yunker et al. (20). TC-100 was from GIBCO and was modified according to Lynn (11). TC-199 was modified as described by McIntosh et al. (15) by mixing equal volumes of TC-199 and Melnick's medium A (GIBCO).

Defined lipid concentrate. A chemically defined lipid concentrate from GIBCO was incubated in IPL-52B at various concentrations for 24 h before bioassay as described earlier (1).

Cell culture and preconditioned media. Two of the cell lines originated from imaginal wing discs of *Spodoptera frugiperda* (IAL-SFD1), and *Trichoplusia ni* (IAL TND1), one from fat body of *Lymantria dispar* (IPLB-LdFB),

and two from pupal ovaries of *Helicoverpa zea* (BCIRL-HZ-AM1), and *Heliothis virescens* (BCIRL-HV). The cell line developed from gypsy moth fat body IPLB-LdFB was maintained as described by Lynn et al. (12). The IAL-TND1 and IAL-SFD1 cell lines were maintained in suspension and monolayer cultures, respectively, as previously reported (13,14). The BCIRL-HZ-AMN-1 cells were maintained in the same manner as the IAL-SFD1 cell line, which included removal of the cells from the flask surface by gentle flushing of the medium, whereas the BCRIL-HV cell line required the use of a rubber policeman for removal of the cells before passage. Both cell lines were provided by Dr. A. McIntosch, USDA, ARS, Columbia, MO. Conditioned medium was prepared from the attached monolayer cultures by replacing the culture medium with test medium for 22 to 24 h, and then removing the newly conditioned medium for filtering and storage. Cells from suspension cultures were prepared by centrifugation in an IEC tabletop centrifuge at 110 *g*. The supernatant was removed and replaced with the test medium, which was incubated with the cells for 22 to 24 h, as above. In this case, however, the cultures were again centrifuged, and the supernatant, i.e. conditioned medium, was filtered through a Millipore Millex GV syringe filter unit (0.22 μm) and refrigerated until use.

Medium was conditioned with the cell line as described earlier (2). Briefly, cell counts were made with a hemacytometer, the cells were then allowed to settle, and the medium removed. Cells were resuspended at a concentration that equaled 1×10^4 cells/0.1 ml to condition the medium.

Fat body preconditioned media. Twenty milligrams (wet wt.) of fat body from larvae of *S. frugiperda, T. ni, L. dispar, H. zea,* and *H. virescens* was used to condition 100 μl of medium at 26° C for 20 h. We compared the developmental response obtained with medium that was preconditioned with the fat body-derived cell line vs. host larval fat body. To do this we determined the percentage of fat body and blood volumes at various larval weights (450 to 700 mg/larva) to compare the bioassay response with larval equivalents of fat body. Fat body was dissected from 5th instar larvae as described earlier and weighed to determine the percentage of fat body per larva (1).

Collection of eggs and bioassay. Details of the egg collection procedure and bioassay were as previously described (2). Briefly, parasite eggs were dissected from third instar host larvae that were exposed to 3- to 5-day old female wasps for a 2-h period, the eggs thus being less than 4 h old. The eggs were rinsed 5 times in IPL-52B, then 5 eggs were transferred to 100 μl of filter sterilized test medium. Each treatment of 5 eggs per 100 μl of test medium was replicated 3 to 9 times for a total of 15 to 45 eggs. In every bioassay a control containing unconditioned medium was run. In addition, fat body conditioned medium at 20 mg/ml was run as a positive control to ensure that the eggs were capable of responding in the bioassay. Development was observed and recorded for 7 days at 26.6° C. Germ band formation was selected as a marker for early egg development. Data were presented as the percentage of eggs that attained germ band stage and that hatched into first instar larvae. A weighted analysis of variance of the data was calculated using PROC GLM of the SAS computer package. Separation of means was done using the Waller-Duncan procedure (18).

Results

Media comparison. The percentage of pregerm eggs that developed to germ band stage and hatched into first instar larvae in two media conditioned with five cell lines is shown in Table 1. The cell lines tested were all derived from Lepidoptera. In comparing the IPL 52B medium with Grace's medium for each of the cell lines, we found that all of the cell lines supported development to the germ band stage in both media; however, only Grace's medium conditioned with the IAL-SFD1 cell line resulted in a significantly higher percentage of germ band formation than IPL-52B medium cultured with the same cell line. Additionally, eggs in unconditioned Grace's medium developed to the germ band stage, and a small percentage hatched (<4%). In comparing the two media with regard to the percentage of eggs that developed and hatched, IPL-52B media only supported development into first instar parasitoid larvae when conditioned with two of the cell lines IAL-TND1 and BCRIL-HV; however, the percent hatch (<8%) was low with both cell lines. Grace's medium conditioned with the IAL-SFD1, IAL-TND1, IPLB-LdFB, and BCRIL-HV cell lines in Grace's supported development to first instar larvae. The BCIRL-HZ-AM1 cell line did not support development to first instar larva in either IPL-52B or Grace's medium.

TABLE 1

EGG DEVELOPMENT IN MEDIA CONDITIONED WITH CELL LINES AND FAT BODY[a]

Cell Lines	Medium[b] IPL-52B Germ Band (Percent)	IPL-52B Percent Hatch (Percent)	Grace's Germ Band (Percent)	Grace's Percent Hatch (Percent)
Control	$0 \pm 0^{c;A}$	$0 \pm 0^{a;A}$	$45 \pm 6^{a;B}$	$4 \pm 3^{c;B}$
IAL-SFD1	$20 \pm 0^{bc;A}$	$0 \pm 0^{a;A}$	$55 \pm 10^{a;B}$	$17 \pm 13^{bc;A}$
IAL-TND1	$33 \pm 13^{b;A}$	$8 \pm 7^{a;A}$	$47 \pm 7^{a;A}$	$47 \pm 7^{a;B}$
IPLB-LdFB	$57 \pm 10^{a;B}$	$0 \pm 0^{a;A}$	$50 \pm 15^{a;A}$	$43 \pm 12^{ab;B}$
BCIRL-HZ-AMN-1	$10 \pm 7^{c;A}$	$0 \pm 0^{a;A}$	$33 \pm 13^{a;A}$	$0 \pm 0^{c;A}$
BCIRL-HV	$17 \pm 10^{bc;A}$	$3 \pm 3^{a;A}$	$40 \pm 7^{a;A}$	$23 \pm 11^{abc;A}$

[a] Percentage of pregerm band eggs attaining germ band stage and hatching in IPL-52B and modified Grace's media preconditioned for 24 h with the following cell lines: Sf, IAL-SFD1; Tn, IAL-TND1; Ld, IPLB-LdFB; Hz, BCIRL-HZ-AM1; and Hv, BCIRL-HV.

[b] All values are means ± SE of pregerm band eggs developing to germ band stage and hatching into first instar larvae. Within a column, means followed by the same lower case letter are not significantly different ($P > 0.05$). Within a row, the percentage of eggs attaining germ band stage or hatch in IPL-52B is compared with the respective stage in Grace's; in this case, means followed by similar upper case letters are not significantly different ($P > 0.05$).

When the BCIRL-HZ-AM1 cell line was cultured in TC-100, ExCell-400, and TC-199 media, all three media promoted germ band formation (Fig. 1 *A*). Although the BCIRL-HZ-AM1 cell line did not support development of the parasitoid eggs to the first instar larva in either IPL-52B or Grace's medium, it did promote a low percentage of the eggs (<10%) to develop to the first larval instar in two of three other media tested (Fig. 1 *B*). None of the eggs cultured in the TC-100 medium hatched whereas, a low level of hatch (10%) occurred in TC-199 and Excell-400 media. In addition, the TC-100 and TC-199 control media (Fig. 1 *A*), similar to Grace's control medium (Table 1, control), both supported development to the germ band stage without being conditioned with the cell lines. In contrast, unconditioned IPL-52B medium did not support development to the germ band stage (Table 1, control). However, pregerm band eggs did develop to germ band stage in unconditioned IPL-52B medium when a defined lipid concentrate was added to the medium (Fig. 2).

Comparison of Cell Lines In IPL-52B and Grace's Media

Germ band formation. Relative to the other cell lines, IPLB LdFB resulted in a significantly higher number of eggs (57%) developing to germ band stage in IPL-52B medium (Table 1). The BCRIL-HV, IAL-SFD1, and IAL-TND1 (17, 20, and 33%, respectively) were not significantly different from each other, but were all significantly different from the BCRIL-HZ-AM-1 cell line (10%) and control (0%). In Grace's medium, none of the cell lines was significantly different from the control or each other.

Hatch. None of the cell lines conditioned in IPL-52B media was significantly different from each other or the control media (Table 1). Only the IAL-TND1 and HV cell lines (47 and 43%,

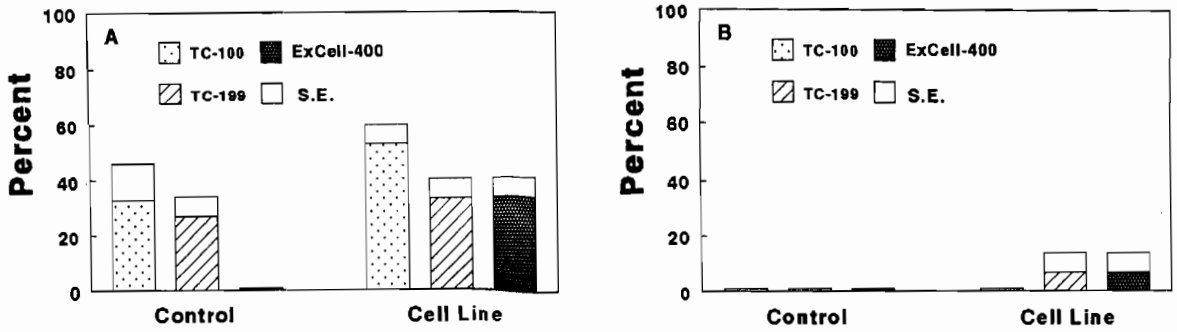

FIG. 1. Percentage of pregerm band eggs attaining germ band stage (*A*) and hatching (*B*) in TC-100, Excell-400, and TC-199 media preconditioned with the cell line BCIRL-HZ-AM1 for 24 h.

respectively) conditioned in Grace's media were significantly different from the control media (4%). The IPLB-LdFB (43%) cell line, however, was not significantly different from the BCRIL-HV (23%) or IAL-SFD1 (17%) cell lines.

Comparison of fat body and cell line conditioned media. A comparison of fat body conditioned media with cell line conditioned media is shown in Fig. 3. This experiment revealed that the embryotrophic activity of the fat body tissue in Grace's medium rather than in IPL-52B was a better predictor of how well cell lines would promote development of *M. croceipes*. For example, in IPL-52B medium the fat body from *T. ni* and *H. zea* stimulated hatch of *M. croceipes*, whereas, the cell lines derived from these species, IAL-TND1 and BCIRL-HZ-AM1, were not effective in promoting development of the parasitoid (Fig. 3). Conversely, in all cases where the fat body stimulated hatch in Grace's medium, the comparable cell line also promoted hatch. These data revealed that the embryotrophic activity of the fat body tissue in Grace's medium rather than in IPL-52B was a better predictor of how well cell lines would promote development of *M. croceipes*.

DISCUSSION

To date, it has not been possible to rear Hymenopteran endoparasitoids from egg to adult in vitro. Some progress, however, has been made in supporting larval development of other species of endoparasites in vitro. An aphid endoparasitoid *Lysiphlebus fabarum* (Hymenoptera: Braconidae) was explanted from the host in the first larval instar and reared to the adult stage in two media conditioned with a cell line derived from *Ceratitis capitata* (Diptera, Trypetidae) (17). Attempts to culture pregerm eggs of *L. fabarum*, however, were unsuccessful. A similar case was observed with two other endoparasitoids. The endoparasitoid *Biosteres longicaudaus* (Hymenoptera:Braconidae) was reared from postgerm band eggs excised from the host fruit fly *Anastrepha suspensa* (Diptera: Trephritidae) to the adult (8). Likewise, *Cardiochiles nigriceps* (Hymenoptera: Braconidae) could be reared from postgerm band eggs to second instar larvae in medium supplemented with 20% fetal bovine serum and other undefined components. Eggs explanted from the host before they had reached germ band stage did not develop in vitro (16). In contrast, we reported that eggs of *Microplitis croceipes* could be explanted from host larvae in the pregerm band stage (within 4 h after oviposition) and reared to the first instar larva in IPL-52B medium conditioned with a fat body cell line IPL-LdFB (2).

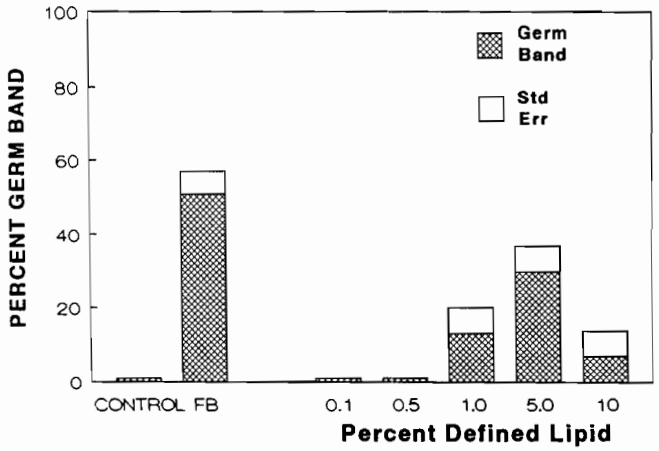

FIG. 2. Percentage of pregerm band eggs attaining germ band stage and hatching in IPL-52B medium containing a chemically defined lipid concentrate; *Control*, IPL-52B medium; *FB*, IPL-52B medium conditioned with fat body from *H. zea* larvae. (From ref. 1).

The present studies were undertaken to compare the capacities of culture medium conditioned with several lepidopteran-derived cell lines to promote embryonic development of a Hymenopteran endoparasitoid. In addition, we investigated the growth-promoting capacities of the cell line in media commonly used to culture insect cells and tissues. The type of medium used to culture the parasitoid eggs immediately after their dissection from host larvae had a substantial effect on their development.

The differences in response to the cell lines could have resulted from their different tissues of origin or because they were derived from different species. Therefore, we compared cell line conditioned media with fat body conditioned media using fat body taken from the same species from which the cell lines were derived (Fig. 3). These data revealed that the embryotrophic activity of the fat body tissue in Grace's medium rather than in IPL-52B was a better predictor of how well cell lines would promote development of *M. croceipes*. However, the fact that host larval fat body seemed to provide the major factor or factors needed for embryogenesis of the parasitoid does not mean that the cell lines derived from tissue sources other than fat body are not capable of providing the same stimulating effect in vitro. The two imaginal wing disc-derived cell lines (IAL-SFD1 and IAL-TND1) as well as the two lines derived from pupal ovaries (BCIRL-HZ-AM1 and BCIRL-HV) all supported embryonic development and hatch into first instar larvae. Thus these studies indicated that the activity and specificity of the in

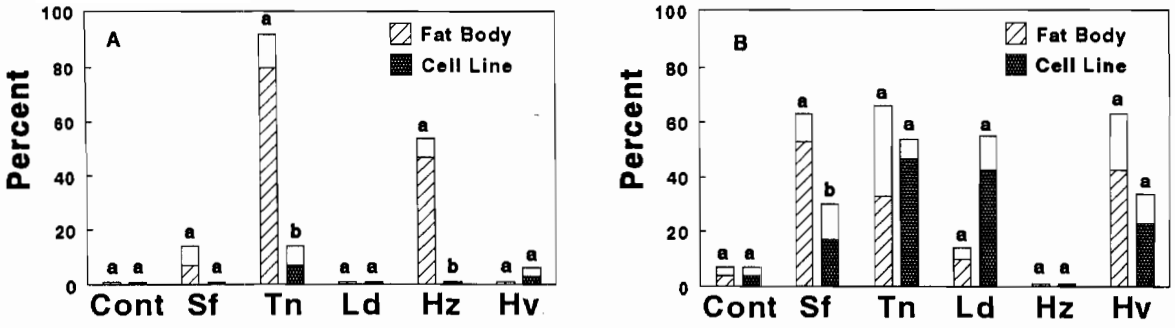

FIG. 3. Percentage of pregerm band eggs hatching in (*A*), IPL-52B and (*B*), modified Grace's media preconditioned for 24 h with fat body from *Sf, Spodoptera frugiperda; Tn, Trichoplusia ni; Ld, Lymantria dispar; Hz, Helicoverpa zea; Hv, Heliothis virescens* or the following cell lines: *Sf*, IAL-SFD1: *Tn*, IAL-TND1; *Ld*, IPLB-LdFB; *Hz*, BCIRL-HZ-AM1; and *Hv*, BCIRL-HV.

vitro conditions on *M. croceipes* egg development depended on both the nature of the cell line and the composition of the medium.

None of the parasitoid eggs cultured in the cell line or fat body conditioned resulted in a rate of growth as high as that reported in vivo. For example, the IPLB-LdFB and IAL-TND1 cell lines induced the highest percentage of eggs to hatch (Table 1), but the time required to reach first instar was an average of 5 to 6 days in both the cell line and host fat body conditioned medium compared to 1.5 to 2 days in vivo (10). Furthermore, first instar larvae did not molt into second instar larvae. In related work it was found that molting of first instar larvae of *M. croceipes* into second instar larvae in vitro was size-dependent and did not depend on hormonal changes (Danise Coar and Patrick Greany, personal communication, Dec 1992). First instar larvae dissected from the host when they were at least 3.0 mm in length, molted into second instar larvae when placed in vitro. Thus, it is plausible that development of a medium that would support greater growth would solve the problem of inducing the larvae to molt. The results of our research on the potential of several cell lines to support the growth and development of *M. croceipes* in vitro indicate that cell line products may be useful in promoting parasite growth in tissue culture, but that other factors need to be identified to optimize the growth and development of *M. croceipes* so that larval molting can be induced. It is interesting that the lipid supplement stimulated germ band formation. We tested the lipid supplement in IPL-52B earlier (5) because phospholipid supplementations have been shown to be mitogenic in certain cell culture systems. However, we do not know whether the germ-band-promoting material released by the cell lines is lipoidal. Studies of the nature of the factor(s) released by the fat body cell line that were responsible for stimulating egg development were initiated earlier (1). Identification and isolation of these factors could be used in developing chemically defined media for growth of the parasitoids.

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